Light Microscopy Core Facility
DUKE UNIVERSITY AND DUKE UNIVERSITY MEDICAL CENTER

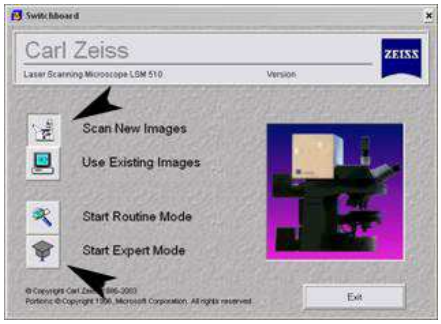
Zeiss LSM 510 inverted confocal manual

Power Up

1. Turn on the mercury lamp (if required)
2. PC/system
3. Components
4. Logon to ASADMIN

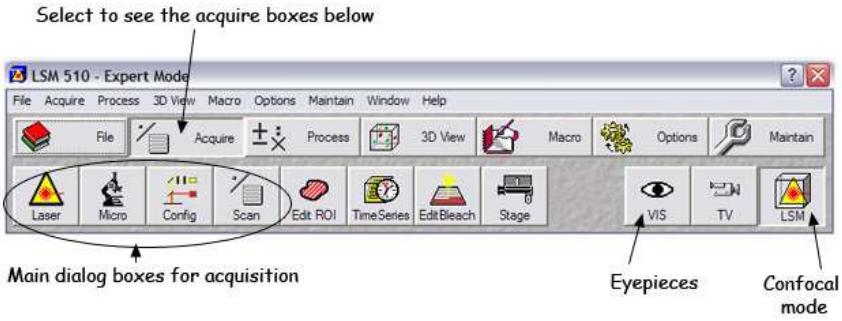
Open the Software

Double-click the 510 icon on the desktop, the LSM switchboard appears . . .



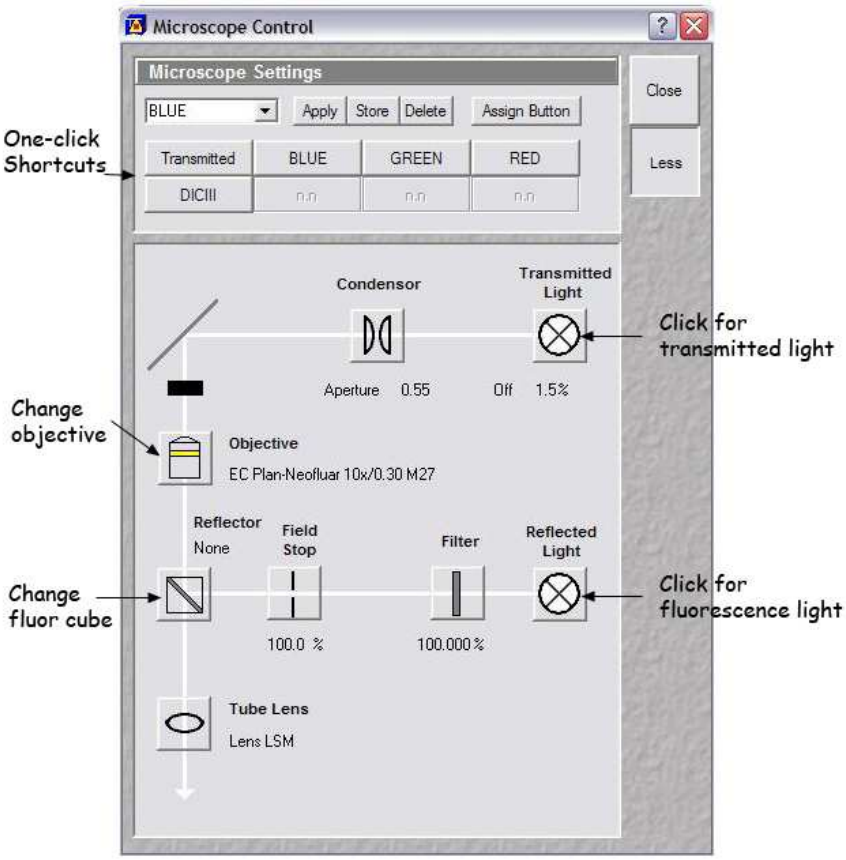
- **Make sure Scan New Images is selected.** ("Use Existing Images" is an off-line emulation mode)
- Click on Start Expert in the Switchboard.

And the software looks like this. . .



Finding Your Specimen in the Eyepieces

The objectives, fluorescent filters, condenser, neutral density filters, and field diaphragm on the Axio Observer stand are motorized and can be controlled in the software via the **Acquire>Micro** submenu or the LCD touchscreen. You may want to find and view your specimen under transmitted light or fluorescence, or both.

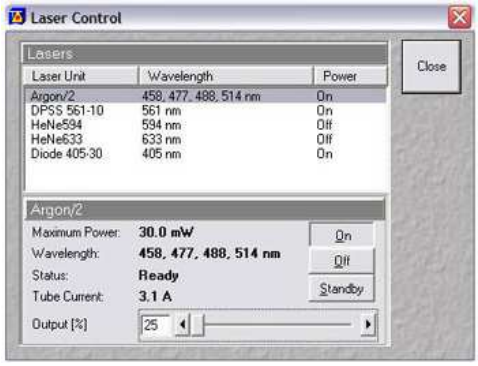


When you've located something you'd like to confocal, click the LSM button on the software panel.

Acquiring a confocal image

Turn On the Lasers

Select **Acquire>Laser** submenu will open the "Laser Control" window with a list of available lasers.



Laser	Lines (nm)	Colour of fluorophores	Examples of fluorophores
405 Diode	405	Deep blue	DAPI
Argon	458 477 **488** 514	Green (Cyan and Yellow)	GFP, Alexa 488, FITC, CY2
561 Diode	561	Red	Alexa 568, TRITC, CY3
594 gas	594	Red	Alexa 594, texas red, mCherry
633 gas	633	Far-red	Alexa 633, CY5

Turn on only the laser(s) you need to excite the fluorophores in your sample.

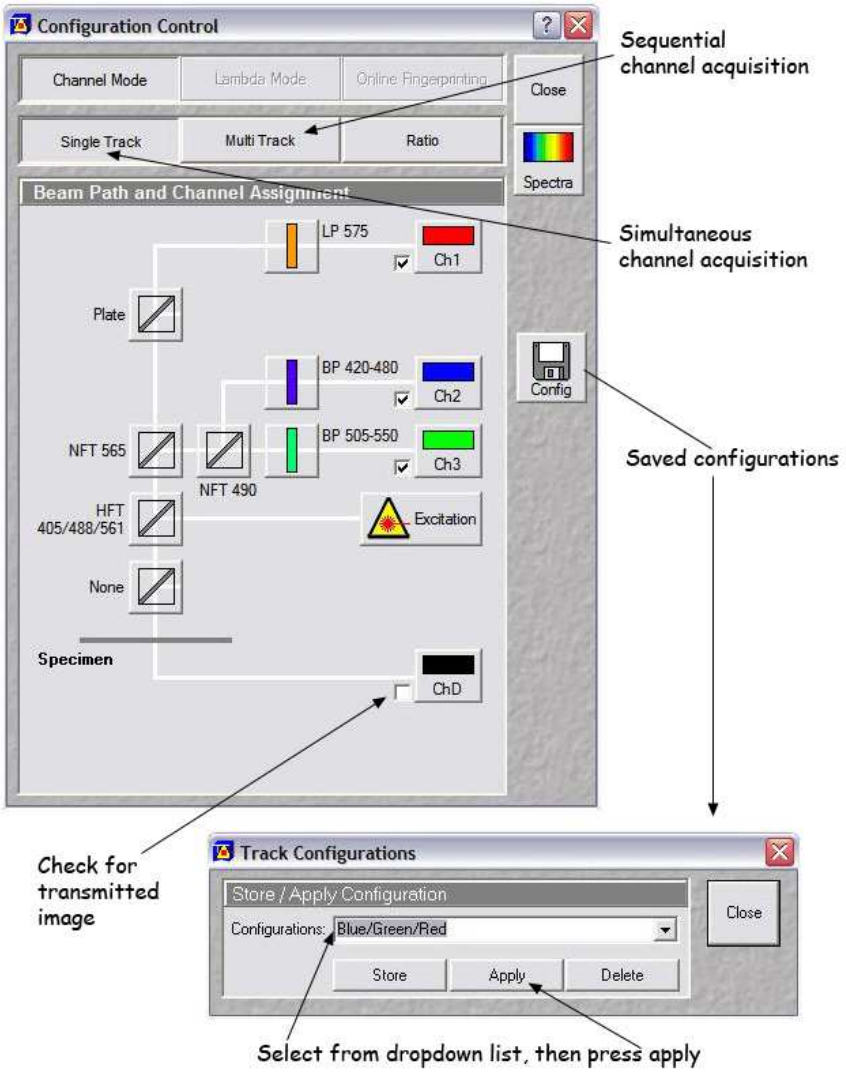
To turn on the Argon laser, click standby to warm it up. After the initial warm-up period, the on button will appear allowing the laser to be fully activated. Set the output intensity to an appropriate level - 40% is a good starting point.

To turn on any of the other lasers, simply click the appropriate "On" buttons - no warm-up is required for ignition.

We try to minimize the number of times a laser is turned on or off - if somebody will use the laser within 2 hours leave it on (Argon laser in standby). Check the reservation schedule to see the laser usage scheduled before and after your session. Turn off any lasers you don't need that may have been left on for you by the previous user, as long as anyone scheduled after you won't use them within 2 hours of your imaging session.

Choose a configuration

A filter configuration for the particular fluorophores you are using can be loaded via the "Configuration Control" window. Open it by selecting the **Acquire>Config** submenu.



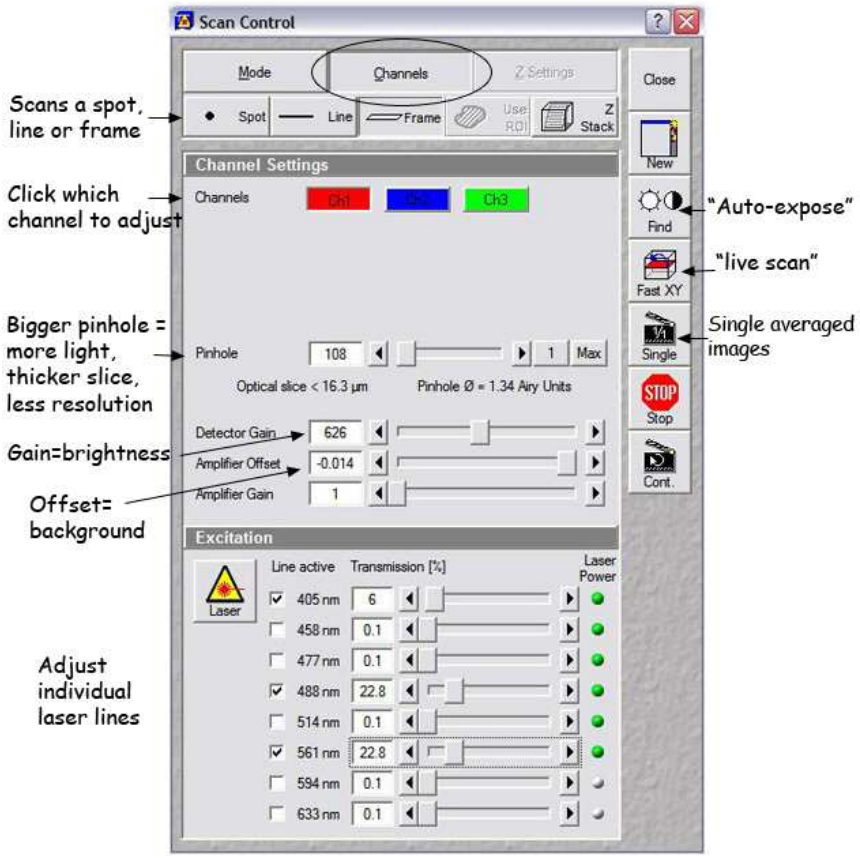
1. **Single- or multi-track?** Single Track mode is for single channel imaging or simultaneous excitation of multiple channels; in Multi Track mode the laser scans multiple channels sequentially, which can be useful when bleedthrough is a problem. Select whichever is appropriate for your imaging needs.

2. **Apply preset configuratio**n The saved configuration button on the right side of the "Configuration Control" window opens the "Track Configurations" window with many popular combinations.

Capture an image: Scan control

Acquire>Scan opens the "Scan Control" window - the main dialog box for acquiring images. There are several adjustments to make to get a good image. Each adjustment may effect the other settings so some interation in the process is required.

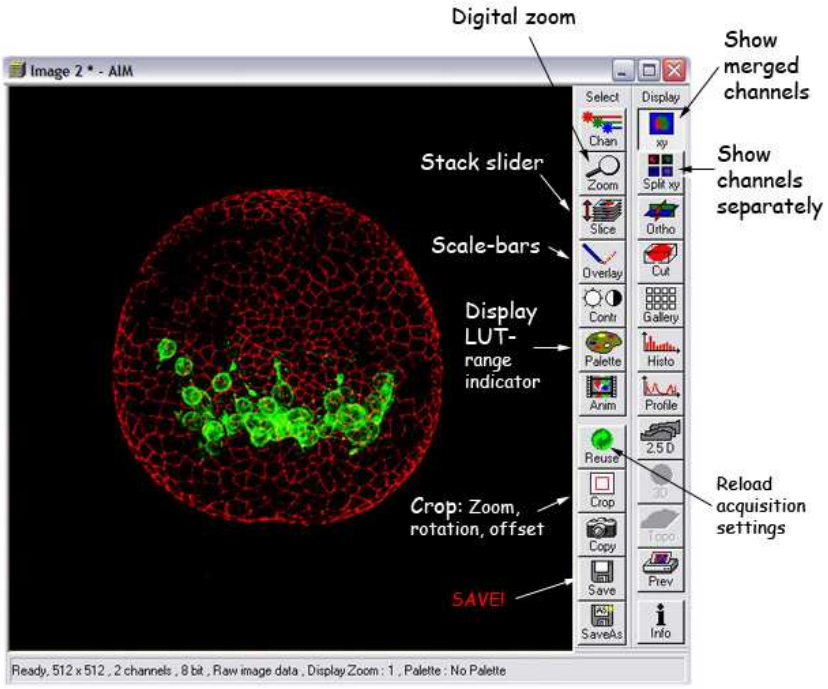
Adjustments under the channels tab . . .



1. Adjust % laser transmission(s) in the Excitation panel at the bottom of the window. Doing so sets the % laser power let through by the AOTF.

2. Set the pinhole diameter with the Pinhole slider scale. The smaller the pinhole, the thinner the optical slice will be and the less light will be collected. A pinhole set to 1 Airy unit (click the 1 button) will have optimal resolution. However, you will probably need to adjust this according to your sample's fluorescence, using larger Airy values for dim samples. For multi-fluorophore imaging, it is usually advisable to set the pinholes for each channel to produce equivalently thick optical slices.

3. Press "Find" to acquire a preliminary image using automatic setting of gain. An image display window will open and the output of the scan will be displayed. If you are performing multi-channel imaging, select Split XY button in the "Image" window's Display toolbar.



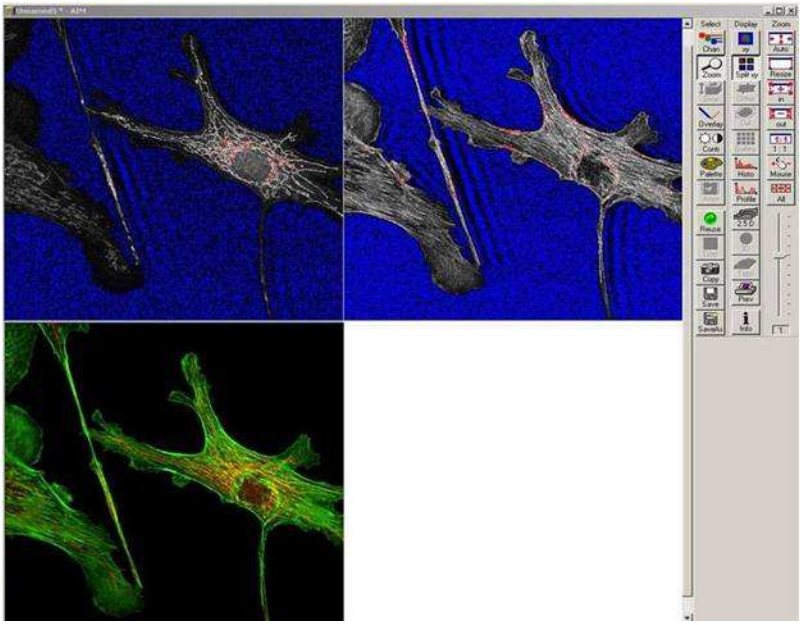
4. Click the Fast XY button and while the laser is scanning, use the XY Stage Controller and **adjust the fine focus on the microscope body.** Clicking "Stop" in the Scan Control window will turn off the Fast XY continuous scanning (minimize bleaching when you can).

The preliminary image, whether too dim or too bright, usually needs to be optimized. The PMTs need to be adjusted according to the fluorescence intensity of the sample.

5. Select each channel separately by clicking on the corresponding coloured button.

6. The range indicator display is helpful for setting the gain and offset. Select Palette in the image window's Select toolbar. From the Color Palette List, select "Range Indicator". In this palette, pixels with intensity of 0 are blue and pixels with intensity of 255 (for 8 bit images) are displayed red.

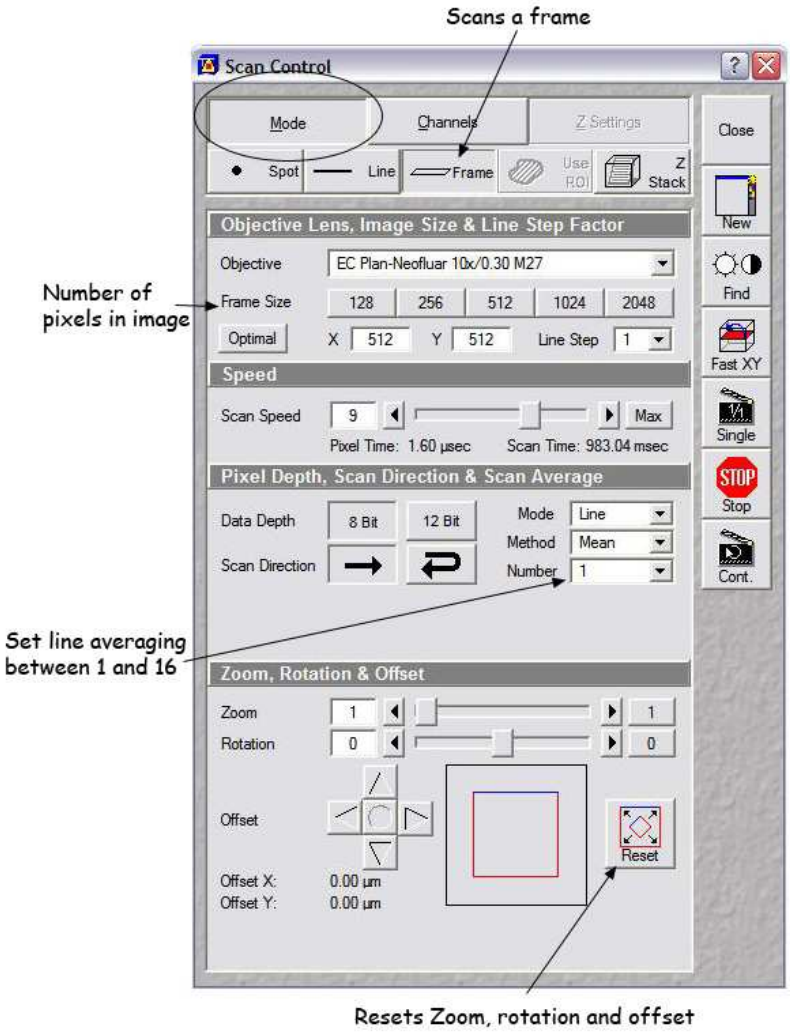
7. Start Fast XY and set the **Detector Gain** until no more than a few % of your stained areas has red pixels. Move the **Amplifier Offset** until the background is a mixture of blue and black pixels. (Always leave the amplifier gain as 1). Make these adjustments for each channel.



8. Reset the Palette to "No Palette".

Adjustments under the mode tab . . .

Most of these will be fine as the default values. Averaging is the most important thing to adjust.



512*512 pixels is good for most imaging, choose other pixel sizes in the "image size" sub-panel

Under Speed, the default scan speed (when not zoomed in) is 9. Reducing the scan speed can improve a noisy image; faster scan speeds are used to find regions of interest (like during the Fast XY function) or when photobleaching of the sample is a concern.

Under Pixel Depth, Scan Direction & Scan Average, select your images to be 8 bit (256 gray levels) or 12 bit (4096 gray levels). Select the Scan Direction to be unidirectional or bidirectional. Unidirectional is typically used, bidirectional scanning can be handy in live imaging since it cuts the scan time in half (but may require phase adjustment).

To **set the averaging for your final image**, choose between line averaging, using the mean intensity of 1,2,4,8, or 16 scans.

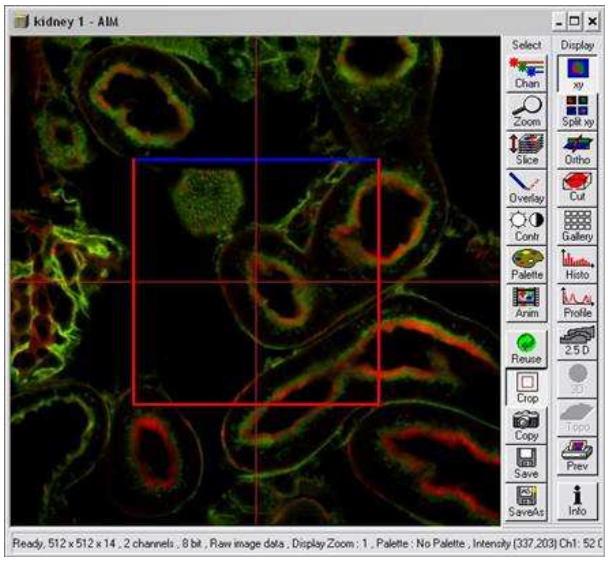
The amount of averaging necessary to obtain a good image depends on the signal to noise ratio of your sample image. Low contrast images will require more averaging but be aware that this could cause photobleaching. You can determine the amount of averaging necessary to yield the best image of your sample empirically. As a rough guide, averaging should be set to 100th the gain value, meaning most samples will require averaging of 4 or 8.

The zoom and scan field rotation can be set in the Zoom, Rotation, & Offset panel (Offset here refers to fine XY movement of the scan area). You may find it more convenient to use the crop function in the "Image" window -

Crop Your Image (optional)

This function is a true optical zoom performed upon image acquisition - the laser will scan a user-defined region of interest and display the scan data in the designated frame size (512x512, etc). It is a convenient way of setting the zoom, rotation and offset displayed under the Mode tab.

Press "Crop" from the Image window display toolbar. A crop box appears in the current image window, which can be moved, rotated and resized with the mouse.



Pressing "single" will rescan with the modified zoom etc settings

(Pressing the "reset" button in the mode panel is useful for undoing all these changes, ready for the next sample.)

Images are overwritten so save as you go along

- Images are saved as **.lsm files** within a **.mdb database.** Images can be exported as TIFFs
- Click the "Save" icon on the bottom right of the image window
- If you don't have a database already, you need to create one
- Make sure you are saving images to the correct database
- Name the image and press save

Users should generally save their images either to their own user folder on the \\Bio-lemming\Confocal fileserver or directly to a USB memory drive. Any images saved on the hard-drives are unsafe and are subject to deletion WITHOUT WARNING. The Imaging Suite anteroom (4226 FFSC) has a PC workstation with both a USB port and a DVD writer that can be used for transferring data off the network to portable storage devices.

Z-sectioning

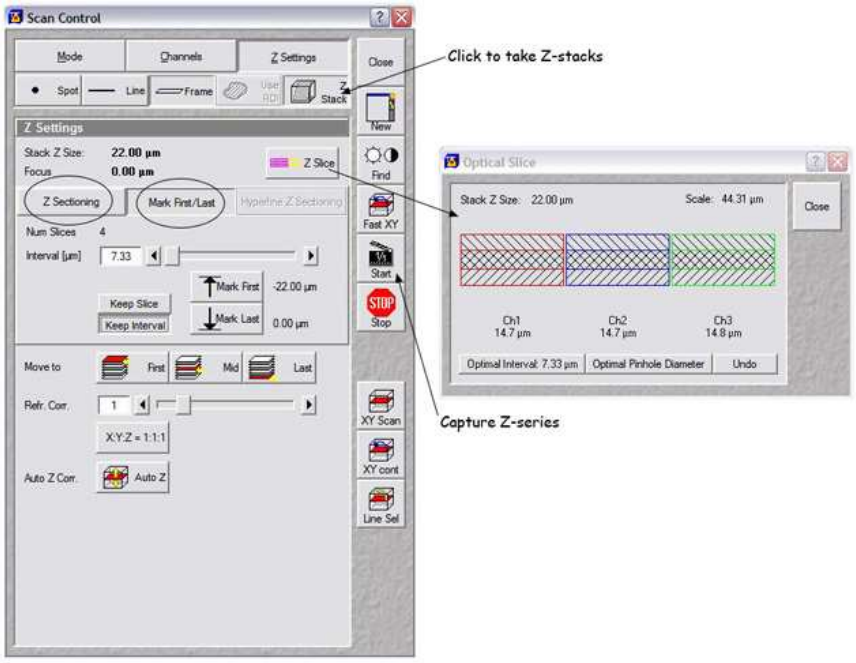
To collect a series of images at different points along the z-axis the settings can be inputted in two ways: Z Sectioning or Mark First/Last.

- Z-sectioning - takes x slices at a particular z interval
- Mark first/last - set the top and bottom of your sample and select either the interval or number of slices (the other is calculated for you)

Both of the methods described here assume that the user has optimized and set all imaging parameters, including pinhole size, detector gain/offset, scan time and averaging described above.

Mark First/Last Method

1. Select Z Stack at the top of the "Scan Control" window - doing this will activate the Z Settings panel.



2. Determine the start and end points of the Z stack. (Z sectioning is performed moving into the specimen, away from the coverslip.) To find the starting point for the Z stack perform a Fast XY scan and focus toward the coverslip (rotate fine focus knob toward you) until you reach the point at which you'd like to begin the Z stack. Stop the Fast XY scanning.

3. Click Mark First in the Mark First/Last tab in the Z Settings panel.

4.Start another Fast XY scan and turn the fine focus knob away from you focusing down through your specimen until you reach the point at which you'd like to end the Z stack. Stop the Fast XY scanning.

5. Click Mark Last to set the end point of the stack.

6. Assuming you have already adjusted your pinholes as described in "Acquire Menu: Collect An XY Section", look up the optimal z interval thickness by clicking Z Slice at the top of the Z Settings box. This will open the "Optimal Slice" window.

7. Optimal z interval thickness for the objective currently in use will be displayed in this window. Clicking Optimal Interval in the lower left corner of the window will set the z interval of your z stack to the recommended thickness.

8. Start will begin scanning of the z stack. Settings last entered in the Channels and Mode panels will be applied to this z stack collection.

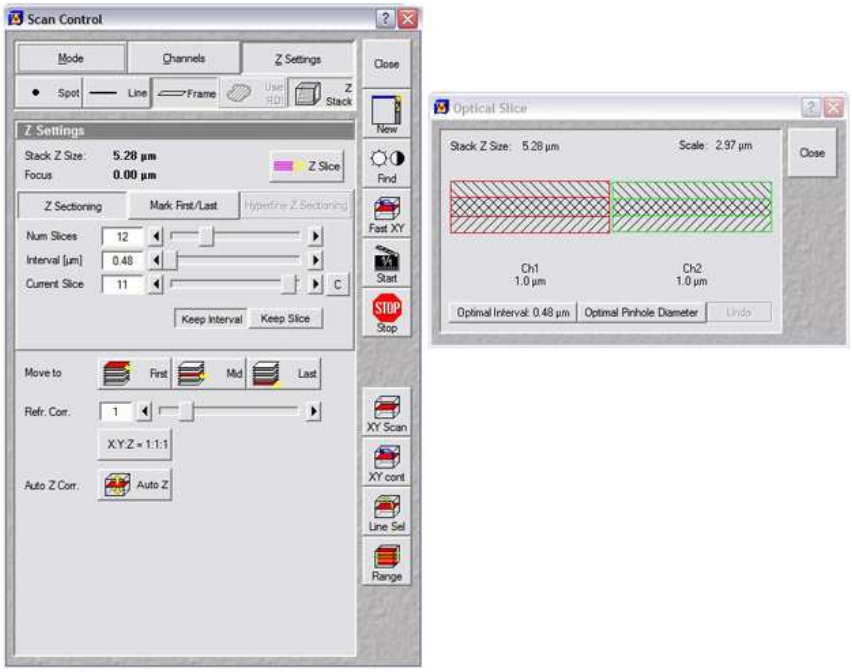
9. The collected z stack is temporarily stored in the computer's memory. To permanently store the stack, use Save or Save As in the image display window to save it to a database.

Z Sectioning Method

1. This method assumes the user has prior knowledge of the specimen's thickness and/or is willing to guesstimate thickness. Select Z Stack at the top in the "Scan Control" window - doing this will activate the Z Settings panel.

2. Click on the Z Sectioning tab and select Keep Interval. This will keep the z slice size constant, even if you later decide to change the Num Slices or the upper and lower limits in your stack.

3.Type in the desired interval for your z stack in the **Interval (um)** field of the Z Sectioning tab. If necessary, look up the optimal z interval thickness by clicking Z Slice at the top of the Z Settings box. This will open the "Optimal Slice" window. Assuming you have already adjusted your pinholes as described in "Acquire Menu: Collect An XY Section", your optimal z interval thickness for the objective currently in use will be displayed in this window. Clicking Optimal Interval in the lower left corner of the window will set the z interval of your z stack to the recommended thickness.



4. The **Num Slices** will be calculated automatically based on the chosen interval input.

5. Designate your current focal position (if you're in focus, you're probably somewhere near the middle of the specimen) in the **Current Slice** field.

6. Clicking Start will begin scanning of the z stack. Settings last entered in the Channels and Mode panels will be used to collect this z stack collection.

7. The collected z stack is temporarily stored in the computer's memory. To permanently store the stack, use Save or Save As in the image display window to save it to a database.

Time series

The Time Series is a versatile software function that is useful in a variety of situations - from collecting single confocal images or entire z stacks over time to more sophisticated applications such as FRAP. If desired, a delay between the beginning of one scan process to the beginning of the next can be set. It is important to note that Time Series works at only one location on the slide. To apply the Time Series function to multiple stage locations, you will have to use the Multitime macro.

The following instructions are for applying a basic Time Series. It is assumed that the user has optimized and set all imaging parameters beforehand (pinhole size, detector gain/offset, scan time, averaging, and if desired, z stack). Refer to previous sections for more details on adjusting these settings.

1. Select the **Acquire>Time Series** submenu.

2. In the Start Series panel, choose between a Manual start or a timed start via Time. Most users want a manual start. If a timed start is desired, enter a start time in the Time (h:m:s) field. The time series will start when the set time is reached according to the computer's clock.

3. In the End Series panel, enter the number of time points desired in the **Number** field if a Manual end is selected. For a timed end point, depress Time and enter the end time in the Time (h:m:s) field.

In determining an experiment's length and the number of time points needed it is important to consider the scan time and averaging settings (see the Speed section of the Mode panel in "Scan Control"). Both parameters influence how long it takes the laser to clear the scan field and thereby define the minimum time between time points.

4. The next panel you encounter will be either Time Interval or Time Delay, depending on the settings of your Time Series register (see **Options--> Settings**). Time Delay is defined as the end of one scan process to the beginning of the next, while Time Interval is the time between the beginning of one scan process to the beginning of the next. Either panel, Time Interval or Time Delay, permits time series scan parameters to be activated or changed from experiment to experiment.

You may load & Apply previously saved time series settings using the pulldown menu in either of these panels. Or you may choose to manually enter scan parameters into the **Time** and **Unit** fields. If you foresee performing other experiments with identical time scan settings, you should Store your manually entered settings to the dropdown list for easy reference in the future.

5. Click Start T in the right-hand side of the "Time Series" window to start your time series.

6. The collected time series is temporarily stored in the computer's memory. To permanently store the series, use Save or Save As in the image display window to save it to a database.

Ending your session

Remove your sample from the stage and clean any oil objectives you used. Please let us know of any problems, the Report Problem form at http://microscopy.duke.edu/problem.html is quick to use.

Check the on-line reservation schedule to see if/when the next reservation is scheduled:

If someone else is scheduled to use the microscope within 2 hours...

- Put the Argon laser into standby and turn off any lasers that won't be used by the next person, leaving on hte ones indicated on the booking calendar
- Exit the software
- Select the "Log Off" command from the Start menu of the desktop and leave the system on.
- Sign the logbook

If you are the last scheduled session of the day or for more than 2 hours...

- Turn off all the lasers in the **Acquire>laser** box and exit the software
- Select "Shut down computer" command in the Start menu of the desktop. The computer and monitors will power down automatically.
- Turn off the Components switch
- Turn of the PC/system switch
- Turn of the arc lamp
- Sign the logbook

Image Processing & Analysis

Scalebars & Measurements

A scale bar, measurements (e.g. angle, area, line) and/or text can easily be drawn onto an image that has already been collected using the Overlay option in the image display window. You are encouraged to explore the drawing buttons in the Overlay toolbar - creating a scalebar on your image is described here.

1. Click Overlay in the Select toolbar of an open image window.

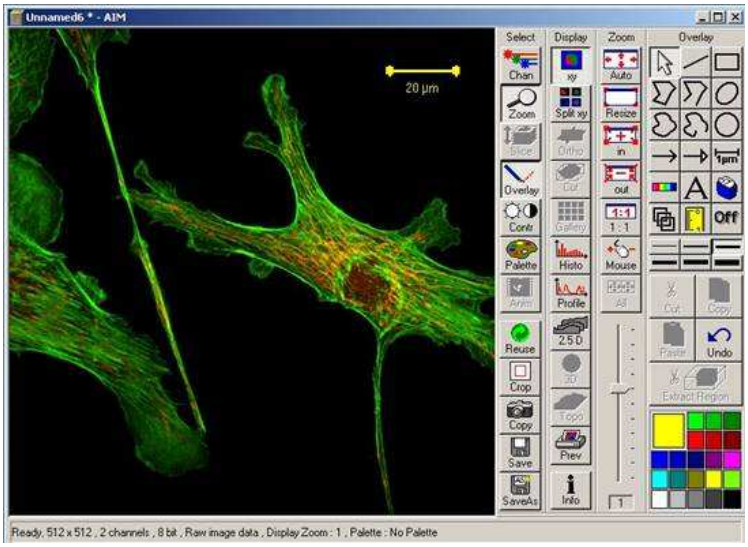
2. Select the desired scalebar thickness from the line width option buttons appearing half way down the Overlay image window submenu.

3. Click on the desired color of your scale bar from the color selection at the bottom of the Overlay submenu.

4. Click 1um in the Overlay submenu, then click in the vicinity of the image where you wish the scalebar to be drawn.

5. Drag the mouse over some distance on the image until the resulting scalebar registers your desired value. Release the mouse button to draw this scalebar overlay on the image.

6. Use Save or Save As in the image window to save it to a database.



3-D stack display options

Animate

Some users will find it helpful to assess their time or z-stack data by animating it.

1. Select Anim from the Select toolbar of a stack's "Image" window. The "Animate" window will open and the stack will immediately be animated.

2. Speed 1 and Speed 2 in the bottom section of the "Animate" window allow the user to control the speed of the animation.

3. To further control the animation, use the function buttons in the top section of this window to animate forward and/or in reverse, stop, fast forward, and fast back through a stack. + and - will animate the stack in forward or reverse by whatever increment you choose in the **Increment** slider in the bottom section of this window.

4. The **Increment** slider reduces the number of slices included in the animation to every nth slice as selected.

Gallery

Stack slices are organized in rows and presented in the order in which they were generated inside the image window. A 2-dimensional gallery of z-stacks over time can also be generated. Each slice's z-data (um) and/or temporal interval can easily be displayed in every tile of the gallery. Z- or t-galleries can be saved as an image in a database for quick future reference. Additionally, a subset of images within a stack can be selected and stored for later use (e.g. to create a 3-D projection).

1. Click Gallery in the Display toolbar of the image window. A gallery of the entire stack is automatically generated and the Gallery toolbar becomes available in the image window. Click Save if you want to save the gallery to your database for quick future reference. Otherwise, a gallery view can always be generated from the stack at a later time.

2. For z-stacks taken over a time series, refer to the Gallery menu to select the format in which you'd like the gallery be displayed - Z , Time , or entire z-stacks over time, Z + Time .

3. To have the stack data (z distance or time interval) displayed in every tile of the gallery, click Data in the Gallery toolbar. Use the color selection button (located below Data) to ascribe a color of your choice to the gallery data display.

4. To separate a subset of images from the full image gallery, select Subset in the Gallery

toolbar. Here, define new start and end points of the series using the **Start Slice** and **End Slice** sliders/data fields, or enter a value in the Every n-th Slice field if you would rather create a subset of every n-th slice of your choosing.

3-D Projection

The Projection function can render a single projection or a series of calculated projections at set angle increments around the X, Y, or Z axis. The user can choose between manipulating aspects of the projection's transparency or creating a projection that has maximum intensity.

The "Transparent" projection method decreases the brightness of each file in the projected stack to reveal structure in subsequent layers that would otherwise be hidden. The "Maximum" projection method takes the intensity values of individual pixels in all sections and collapses them into one single illuminated image. The "Maximum" method is chosen when the maximum pixel intensity is desired to be represented in the projection.

Creating a maximum projection is described here. These instructions assume that an image stack is open and therefore available for projection.

1. Select **3D View> Projection** from the main menu. The Projection window will open.

2. Select the stack to be projected in the Source panel of this window.

Only stacks and images that are currently open are available for selection in the Source pulldown list.

3. To create an en face (straight-on) projection of all slices in the z-axis, select the Y radiobutton in the Projection panel. Leave the First Angle set to 0 degrees, enter 1 in the **Number Projections** field, and set the **Difference Angle** field to 0 degrees.

4. In the Transparency tab, select the Maximum radiobutton.

5. Generate the projection by clicking Apply located in the upper right corner of the Projection window.

6. If a sequence of projections at set angles has been created, it may be animated or viewed in a gallery via the Select and Display toolbars of the image window.

Exporting Files to .tif Format

Files can be exported in a couple of different ways - via the batch export macro, which allows the simultaneous export of multiple database items or the Export of Images function, which allows the export of individual database items - be they single confocal sections or a stack. The Export of Images method is described here.

1. Open your database and load (open) the file you would like to export.

2. Select **File >Export** from the main menu. The "Export Images and Data" window will open.

3. In the **Save In** field, designate to which drive or directory the exported data should be saved.

4. Enter a name for the exported image or series of images in the **File name** field.

5. In the **Save as type** field, select the image format into which the file should be exported.

When selecting an image format, pay heed to whether you are exporting a single confocal image or a series (stack) of confocal images. Various image formats are supported (e.g. .bmp, .jpg), we recommend TIF (.tif) format as it is lossless and widely usable.

For multi-channel images, you have the option of exporting individual color channels of data in

monochrome (grayscale) if Raw data single or Raw data series image type is chosen.

6. Click Save.

When a z- or time stack file is exported, each stack slice is stored as an individual TIF image with a 3-digit numbered suffix appended to the designated file name.

Assigning Macros To Buttons In Macro Control

1. Open the software and click Macro main menu item.

2. Select Macro that now appears in the bottom right corner of the main menu toolbar. This opens the Macro Control window.

3. Click on the Assign Macro to Button panel.

4. Indicate which button you want the new macro assigned (Button1, Button2, Button3, etc) from the drop down menu in the Button field.

5. Name the new button in the Text field.

6. Click the ... button beside the Project field to link a macro of your choice to the newly created button. An Open window appears. All macro programs can be found in the C:AIM\Macros folder.

7. Find the macro file that you want to load. Select this file and click on Ok in the Open window.

8. Select the macro name from the drop down menu of the Macros field.

9. Hit Apply and the macro you selected should be assigned to the button you designated in step 4. In a moment, the specified button will appear in the Macro toolbar.

Deleting A Macro From A Button In Macro Control

1. Working under the Macro main menu item, select Macro that appears in the bottom right corner of the main menu toolbar. This opens the Macro Control window.

2. Click on the Assign Macro to Button panel.

3. Choose which button you want to disassociate from its macro in the drop down menu in the Button field.

4. Press Delete to delete the linking of the macro program to the button. The specified button should then ghost out to a blank n.n format.

Initializing User Software Settings - Running Registry Files

1. Logon to the system using your ASADMIN user name and password.

2. Run all the registry files in the folder on the desktop:

   Double-click each of the files, after each file, you should see a dialog box confirming the registry has been successfully updated. Click "OK" to these messages.

   You will need to rerun LSM-Macros.reg whenever you want to use a newly installed macro (e.g. newer version of multitime).

3. Start the LSM510 software as normal - "Scan New Image" & "Start Expert Mode"

4. In the Options menu, click the Settings submenu. Make the following settings in the different tabbed sections of this submenu.

Program Start:

- Select the configuration you wish the software to load by default every time you log on.

- Un-select "don't show logo"

Save:

- Select the third radio button - at "Create Database" automatically create a subdirectory with the same name as specified database and create dataset image files in that subdirectory.

Temporary Files:

"Use RAM" is fine unless you're doing time lapse, then you may want to set a temporary location, e.g. to your server directory:

h:ASADMIN\Bio-lemming\Confocal\lab name\user name or use "Save To Disk"

Import/Export:

Set location of files; click "User Default Path" to use your default directory defined by your user profile

Record/Reuse:

Check all but objective